EFFECT OF RAF-1 KINASE INHIBITOR ON HUMAN BONE AND SOFT TISSUE SARCOMA CELL LINES

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INTRODUCTION

The mitogen-activated protein kinase (MAPK) signaling pathway is activated by signals from growth factor receptors, and it plays a crucial role in the cell proliferation. Several authors have reported that dysregulation of the MAPK signaling pathway exists in many human malignancies, and it is suggested that most of its critical components will be targets for therapeutic development against human malignancies. Raf-1, which is an essential serine/threonine kinase, is a downstream effector of the central signal transduction mediator Ras in the MAPK signaling pathway (RAF/MEK/ERK pathway), and the therapeutics targeting Raf-1 are undergoing clinical evaluation on some human malignancies. We consider that the antitumor activity will be demonstrated on human bone and soft tissue sarcomas by Raf-1 kinase inhibition. We examined the expression of Raf-1 in human bone and soft tissue sarcoma cell lines, and the inhibitory effect of Raf-1 kinase inhibitor on the cell proliferation.

MATERIALS AND METHODS

Cell lines and reagent.

3 human osteosarcoma cell lines (KTHOS, MG63 and KHOS) and 4 human MFH cell lines (TNMY1, GBS-1, Nara-F, and Nara-H) were used in this study. KTHOS and TNMY1 were previously established in our laboratory. GBS-1 was kindly provided by Dr. H. Kanda (Department of Pathology, The Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan). Nara-F and Nara-H were purchased from ScienStuff Co. (Nara, Japan). All cell lines were grown in culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). The cell lines were routinely maintained at 37°C in a humidified 5% CO_2 atmosphere. GW5074, a specific Raf-1 kinase inhibitor, was purchased from Sigma-Aldrich.

mRNA expression of Raf-1.

Total RNAs were eluted by selective binding to a silica-gel-based membrane using an RNeasy Mini Kit® (QIAGEN Inc., Valencia, CA). Reverse transcription of RNA into cDNA was performed by using Reverse Transcription System (Promega, Madison, WI). Raf-1 and GAPDH mRNA expression were examined by reverse transcription (RT-) PCR. After PCR amplification, 8-μl aliquots of the PCR products were electrophoresed in a 2% agarose gel, followed by ethidium bromide dye.

The inhibitory effect of Raf-1 kinase inhibitor.

The cell proliferation was assayed using the MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI). Cells were seeded in 96-well cell culture plates in culture medium with 10% FBS. After 48 hours (h), the medium was refreshed with 1% FBS containing GW5074 in the indicated concentrations. After 24, 48, 72, 96, and 120 h, the medium was removed and washed with phosphate buffered saline, then refreshed with fresh medium containing MTS reagent. The optical density was measured at 490 nm using an automatic microplate reader after 2 h of further incubation. The percent viability of each well was calculated. At least three independent cultures were performed for each study. The percent viability of each well was calculated. The data were analyzed statistically using ANOVA with Fisher's PLSD post hoc test.

Western blotting.

Cells were pretreated for 60 min with 1% FBS containing GW5074 in the indicated concentrations before stimulation with or without 10 ng/ml PDGF for 10 min. Whole cell lysates were collected for protein content, and cell lysates were separated by SDS polyacrylamide gel electrophoresis under reducing conditions. Then gels were electrophoretically transferred to PVDF membrane, and immunoblotted with anti-Raf-1 antibody (Upstate Biotechnology, Lake Placid, NY) and anti-phospho-Raf-1 antibody (Upstate Biotechnology). Bound antibodies were detected using the ECL plus western blotting detection system (GE Healthcare Bio-Sciences, Piscataway, NJ).

RESULTS

mRNA expression of Raf-1.

The Raf-1 mRNA was expressed in all osteosarcoma and MFH cell lines. The expression was the strongest in TNMY1, and the weakest in GBS-1 (Fig. 1).

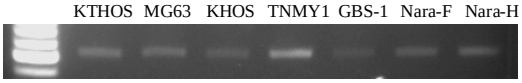

Fig. 1. RT-PCR of Raf-1

The effect of the specific Raf-1 kinase inhibitor GW5074

GW5074 inhibited the cell proliferation of all 7 cell lines in a dose- and time-dependent manner. 10μM GW5074 inhibited the cell proliferation of KTHOS, KHOS and TNMY1 at the percent viability of 50% or less. The cell proliferation inhibition by GW5074 in GBS-1 and Nara-F was lower than in that KTHOS, KHOS and TNMY1 (Fig.2).

Expression of Raf-1 and phospho-Raf-1 kinases.

Western blotting analysis revealed that not only Raf-1 but phospho-Raf-1 were expressed in all cell lines under the normal condition (Fig.3), so it was suggested that Raf-1 was always activated in all 7 cell lines under the normal condition. Phosphorylation of Raf-1 were increased by PDGF stimulation in 6 cell lines other than Nara-H, and 10μM GW5074 decreased phosphorylation of Raf-1 in all cell lines.

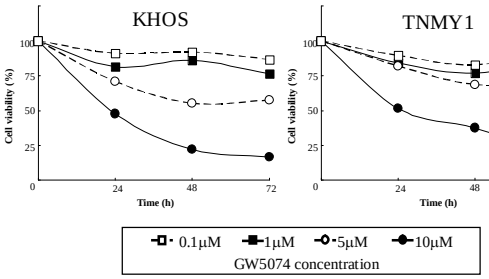

Fig. 2. The inhibitory effect of GW5074 on the cell proliferation of human osteosarcoma and MFH cell lines.

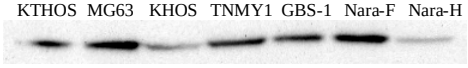

Fig. 3. Western blotting of phosphor-Raf-1 at the normal condition

DISCUSSION

The MAPK pathway is very important as a target of the molecular targeting therapy. Sorafenib (BAY 43-9006, Nexavar((R))), a selective Raf kinase inhibitor that targets RAF/MEK/ERK pathway, has recently been approved by the FDA for advanced renal cell carcinoma in phase III clinical trials. In our study, Raf-1 kinase inhibitor GW5074 showed a dose- and time- dependent inhibitory effect on the cell proliferation of human osteosarcoma and MFH cell lines, and decreased the phosphorylation of Raf-1 in a dose-dependent mannar. These results suggest that GW5074 may be a selective inhibitor of Raf-1 kinase in human osteosarcoma and MFH cells. Although further studies are needed to explore the precise molecular mechanisms for the inhibitory effect of Raf-1 kinase inhibitor on cell proliferation in human osteosarcoma and MFH cells, Raf-1 kinase inhibitor will be a potent chemotherapeutic agent for human bone and soft tissue sarcomas.

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